# THE GROWTH OF BACTERIOPHAGE

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## INTRODUCTION

Certain large protein molecules (viruses) possess the property of multiplying within living organisms. This process, which is at once so foreign to chemistry and so fundamental to biology, is exemplified in the multiplication of bacteriophage in the presence of susceptible bacteria.

Bacteriophage offers a number of advantages for the study of the multiplication process not available with viruses which multiply at the expense of more complex hosts. It can be stored indefinitely in the absence of a host without deterioration. Its concentration can be determined with fair accuracy by several methods, and even the individual particles can be counted by d'Herelle's method. It can be concentrated, purified, and generally handled like nucleoprotein, to which class of substances it apparently belongs (Schlesinger (1) and Northrop (2)). The host organism is easy to culture and in some cases can be grown in purely synthetic media, thus the conditions of growth of the host and of the phage can be controlled and varied in a quantitative and chemically well defined way.

Before the main problem, which is elucidation of the multiplication process, can be studied, certain information regarding the behavior of phage is needed. Above all, the "natural history" of bacteriophage, *i.e.* its growth under a well defined set of cultural conditions, is as yet insufficiently known, the only extensive quantitative work being that of Krueger and Northrop (3) on an anti-*staphylococcus* phage. The present work is a study of this problem, the growth of another phage (anti-*Escherichia coli* phage) under a standardized set of culture conditions.

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## EXPERIMENTAL

*Bacteria Culture.*—Our host organism was a strain of *Escherichia coli*, which was kindly provided by Dr. C. C. Lindegren. Difco nutrient broth (pH 6.6–6.8) and nutrient agar were selected as culture media. These media were selected for the present work because of the complications which arise when synthetic media are used. We thus avoided the difficulties arising from the need for accessory growth factors.

*Isolation, Culture, and Storage of Phage.*—A bacteriophage active against this strain of *coli* was isolated in the usual way from fresh sewage filtrates. Its homogeneity was assured by five successive single plaque isolations. The properties of this phage remained constant throughout the work. The average plaque size on 1.5 per cent agar medium was 0.5 to 1.0 mm.

Phage was prepared by adding to 25 cc. of broth, 0.1 cc. of a 20 hour culture of bacteria, and 0.1 cc. of a previous phage preparation. After 3½ hours at 37° the culture had become clear, and contained about $10^9$ phage particles.

Such lysates even though stored in the ice box, decreased in phage concentration to about 20 per cent of their initial value in 1 day, and to about 2 per cent in a week, after which they remained constant. Part of this lost phage activity was found to be present in a small quantity of a precipitate which had sedimented during this storage period.

Therefore, lysates were always filtered through Jena sintered glass filters (5 on 3 grade) immediately after preparation. The phage concentration of these filtrates also decreased on storage, though more slowly, falling to 20 per cent in a week. However, 1:100 dilutions in distilled water of the fresh filtered lysates retained a constant assay value for several months, and these diluted preparations were used in the work reported here, except where otherwise specified.

This inactivation of our undiluted filtered phage suspensions on standing is probably a result of a combination of phage and specific phage inhibiting substances from the bacteria, as suggested by Burnett (4, 5). To test this hypothesis we prepared a polysaccharide fraction from agar cultures of these bacteria, according to a method reported by Heidelberger *et al.* (6). Aqueous solutions of this material, when mixed with phage suspensions, rapidly inactivated the phage.

*Method of Assay.*—We have used a modification of the plaque counting method of d'Herelle (7) throughout this work for the determination of phage concentrations. Although the plaque counting method has been reported unsatisfactory by various investigators, under our conditions it has proven to be entirely satisfactory.

Phage preparations suitably diluted in 18 hour broth cultures of bacteria to give a readily countable number of plaques (100 to 1000) were spread with a bent glass capillary over the surface of nutrient agar plates which had been dried by inverting on sterile filter paper overnight. The plates were then incubated 6 to 24 hours at 37°C. at which time the plaques were readily distinguishable. The 0.1 cc. used for spreading was completely soaked into the agar thus prepared in

2 to 3 minutes, thus giving no opportunity for the multiplication of phage in the liquid phase. Each step of each dilution was done with fresh sterile glassware. Tests of the amount of phage adhering to the glass spreaders showed that this quantity is negligible.

The time of contact between phage and bacteria in the final dilution before plating has no measurable influence on the plaque count, up to 5 minutes at 25°C. Even if phage alone is spread on the plate and allowed to soak in for 10 minutes, before seeding the plate with bacteria, only a small decrease in plaque count is apparent (about 20 per cent). This decrease we attribute to failure of some phage particles to come into contact with bacteria.

Under parallel conditions, the reproducibility of an assay is limited by the sampling error, which in this case is equal to the square root of the number of plaques (10 per cent for counts of 100; 3.2 per cent for counts of 1000). To test the effect of phage concentration on the number of plaques obtained, successive dilutions of a phage preparation were all plated, and the number of plaques enumerated. Over a 100-fold range of dilution, the plaque count was in linear proportion to the phage concentration. (See Fig. 1.)

Dreyer and Campbell-Renton (8) using a different anti-*coli* phage and an anti-*staphylococcus* phage, and a different technique found a complicated dependence of plaque count on dilution. Such a finding is incompatible with the concept that phage particles behave as single particles, *i.e.* without interaction, with respect to plaque formation. Our experiments showed no evidence of such a complicated behavior, and we ascribe it therefore to some secondary cause inherent in their procedure.

Bronfenbrenner and Korb (9) using a phage active against *B. dysenteriae* Shiga, and a different plating technique found that when the agar concentration was changed from 1 per cent to 2.5 per cent, the number of plaques was reduced to 1 per cent of its former value. They ascribed this to a change in the water supplied to the bacteria. With the technique which we have employed, variation of the agar concentration from 0.75 per cent to 3.0 per cent, had little influence on the number of plaques produced, though the size decreased noticeably with increasing agar concentration. (See Table I.)

Changes in the concentration of bacteria spread with the phage on the agar plates had no important influence on the number of plaques obtained. (See Table I.) The temperature at which plates were incubated had no significant effect on the number of plaques produced. (See Table I.)

In appraising the accuracy of this method, several points must be borne in mind. With our phage, our experiments confirm in the main the picture proposed by d'Herelle, according to which a phage particle grows in the following way: it becomes attached to a susceptible bacterium, multiplies upon or within it up to a critical time, when the newly formed phage particles are dispersed into the solution.

In the plaque counting method a single phage particle and an infected bacterium containing any number of phage particles will each give only one plaque.

This method therefore, does not give the number of phage particles but the number of loci within the solution at which one or more phage particles exist. These loci will hereafter be called "infective centers." The linear relationship between phage concentration and plaque count (Fig. 1) does not prove that the number of plaques is equal to the number of infective centers, but only that it is proportional to this number. We shall call the fraction of infective centers which

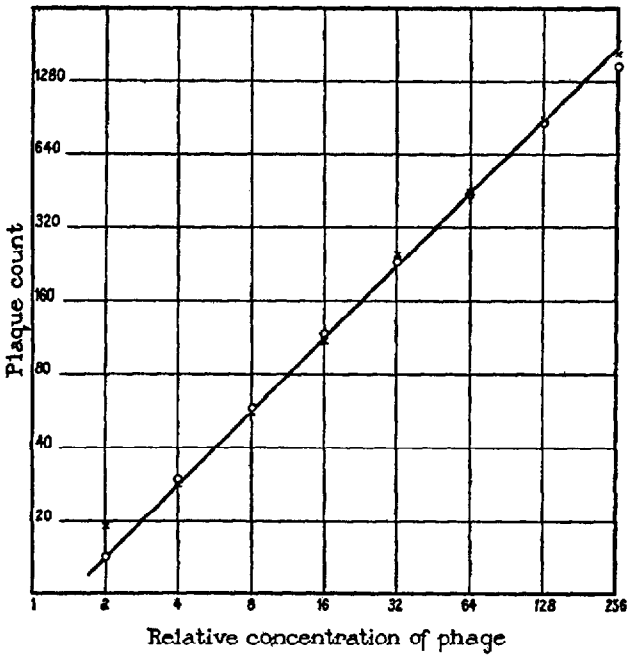


FIG. 1. Proportionality of the phage concentration to the plaque count.

Successive twofold dilutions of a phage preparation were plated in duplicate on nutrient agar; 0.1 cc. on each plate. The plaque counts from two such series of dilutions are plotted against the relative phage concentration, both on a logarithmic scale.

produces plaques the "efficiency of plating." With the concentrations of phage and bacteria which we have used this coefficient is essentially the fraction of infected bacteria in the suspension spread on the plate, which goes through to lysis under our cultural conditions on the agar medium. After plating, the phage particles released by this lysis infect the surrounding bacteria, increasing only the size, and not the number of plaques.

TABLE I

*Independence of Plaque Count on Plating Method*

Agar concentration

Plates were prepared in which the agar strength varied, and all spread with 0.1 cc. of the same dilution of a phage preparation. There is no significant difference in the numbers of plaques obtained.

| Agar concentration, *per cent* | 0.75 | 1.5 | 3.0 |
|---|---|---|---|
| Plaque counts | 394 | 373 | 424 |
| | 408 | 430 | 427 |
| | 376 | 443 | 455 |
| | 411 | 465 | 416 |
| | 373 | 404 | 469 |
| Average | 392 | 423 | 438 |
| Plaque size, *mm* | 2 | 0.5 | 0.2 |

Concentration of plating *coli*

A broth suspension of bacteria ($10^9$ bacteria/cc.) was prepared from a 24 hour agar slant and used at various dilutions, as the plating suspension for a single phage dilution. There are no significant differences in the plaque counts except at the highest dilution of the bacterial suspension, where the count is about 15 per cent lower.

| Concentration | Plaque count |
|---|---|
| 1 | 920 |
| 1/5 | 961 |
| 1/25 | 854 |
| 1/125 | 773 |

*Temperature of plate incubation*

Twelve plates were spread with 0.1 cc. of the same suspension of phage and bacteria, divided into three groups, and incubated at different temperatures. There were no significant differences in the plaque counts obtained.

| Temperature, °C | 37 | 24 | 10 |
|---|---|---|---|
| Plaque count | 352 | 384 | 405 |
| | 343 | 405 | 377 |
| | 386 | 403 | 400 |
| | 422 | 479 | 406 |
| Average | 376 | 418 | 397 |

The experimental determination of the efficiency of plating is described in a later section (see p. 379). The coefficient varies from 0.3 to 0.5. This means that three to five out of every ten infected bacteria produce plaques. The fact that the efficiency of plating is relatively insensitive to variations in the temperature of plate incubation, density of plating *coli*, concentration of agar, etc. indicates that a definite fraction of the infected bacteria in the broth cultures do not readily go through to lysis when transferred to agar plates. For most experiments only the relative assay is significant; we have therefore, given the values derived directly from the plaque counts without taking into account the efficiency of plating, unless the contrary is stated.

### *Growth Measurements*

The main features of the growth of this phage in broth cultures of the host are shown in Fig. 2. After a small initial increase (discussed below) the number of infective centers (individual phage particles, plus infected bacteria) in the suspension remains constant for a time, then rises sharply to a new value, after which it again remains constant. Later, a second sharp rise, not as clear-cut as the first, and finally a third rise occur. At this time visible lysis of the bacterial suspension takes place. A number of features of the growth process may be deduced from this and similar experiments, and this is the main concern of the present paper.

### *The Initial Rise*

When a concentration of phage suitable for plating was added to a suspension of bacteria, and plated at once, a reproducible plaque count was obtained. If the suspension with added phage was allowed to stand 5 minutes at 37°C. (or 20 minutes at 25°C.) the number of plaques obtained on plating the suspension was found to be 1.6 times higher. This initial rise is not to be confused with the first "burst" which occurs later and increases the plaque count 70-fold. After the initial rise, the new value is readily duplicated and remains constant until the start of the first burst in the growth curve (30 minutes at 37° and 60 minutes at 25°).

This initial rise we attribute not to an increase in the number of infective centers, but to an increase in the probability of plaque formation (*i.e.* an increase in the efficiency of plating) by infected bacteria in a progressed state; that is, bacteria in which the phage particle has commenced to multiply. That this rise results from a change in the

efficiency of plating and not from a quick increase in the number of infective centers is evident from the following experiment. Bacteria were grown for 24 hours at 25°C. on agar slants, then suspended in broth. Phage was added to this suspension and to a suspension of bacteria grown in the usual way, and the concentration of infective

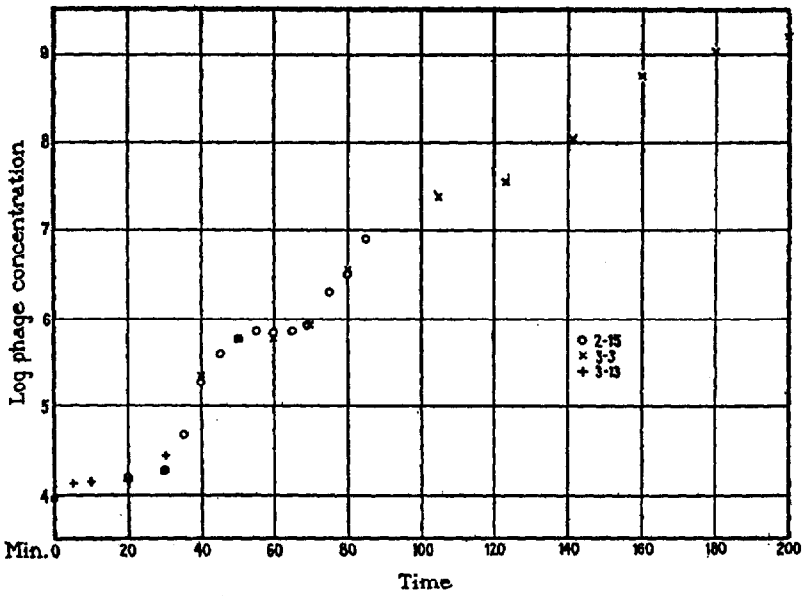


FIG. 2. Growth of phage in the presence of growing bacteria at 37°C.

A diluted phage preparation was mixed with a suspension of bacteria containing $2 \times 10^8$ organisms per cc., and diluted after 3 minutes 1 to 50 in broth. At this time about 70 per cent of the phage had become attached to bacteria. The total number of infective centers was determined at intervals on samples of this growth mixture. Three such experiments, done on different days, are plotted in this figure. The same curve was easily reproducible with all phage preparations stored under proper conditions.

centers was determined on both. The initial value was 1.6 times higher in the agar grown bacteria than in the control experiment, and remained constant until actual growth occurred. The initial rise was therefore absent in this case, clearly a result of an increase in the efficiency of plating. A sufficient number of experiments were per-

formed with bacteria grown on agar to indicate that in other respects their behavior is similar to that of the bacteria grown in broth. The bacteria grown in this way on agar slants are in some way more susceptible to lysis than the broth cultured bacteria.

### *Adsorption*

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. The rate of this attachment can be readily measured by centrifuging the bacteria out of a suspension containing phage, at various times, and determining the amount of phage which remains unattached in the supernatant (*cf.* Krueger (10)).[1]

According to the picture of phage growth outlined above, phage cannot multiply except when attached to bacteria; therefore, the rate of attachment may, under certain conditions, limit the rate of growth. We wished to determine the rate of this adsorption so that it could be taken into account in the interpretation of growth experiments, or eliminated if possible, as a factor influencing the growth rate. Our growth curves show that there is no increase in the number of infective centers up to a critical time; we could therefore, make measurements of the adsorption on living bacteria suspended in broth, so long as the time allowed for attachment was less than the time to the start of the first burst in the growth curve. The adsorption proved to be so rapid that this time interval was ample to obtain adsorption of all but a few per cent of the free phage if the bacteria concentration was above $3 \times 10^7$. The number of bacteria remained constant; the lag phase in their growth was longer than the experimental period.

The rate of attachment was found to be first order with respect to the concentration of free phage ($P_f$) and first order with respect to the concentration of bacteria ($B$) over a wide range of concentrations, in agreement with the results reported by Krueger (10). That is, the concentration of free phage followed the equation

$$-\frac{d(P_f)}{dt} = k_a(P_f)(B)$$

[1] A very careful study of the adsorption of a *coli*-phage has also been made by Schlesinger (Schlesinger, M., *Z. Hyg. u. Infektionskrankh.*, 1932, **114**, 136, 149). Our results, which are less accurate and complete, agree qualitatively and quantitatively with the results of his detailed studies.

in which $k_a$ was found to be $1.2 \times 10^{-9}$ cm.$^3$/min. at 15° and $1.9 \times 10^{-9}$ cm.$^3$/min. at 25°C. These rate constants are about five times greater than those reported by Krueger (10). With our ordinary 18 hour bacteria cultures (containing $2 \times 10^8$ *B. coli*/cc.) we thus obtain 70 per cent attachment of phage in 3 minutes and 98 per cent in 10 minutes. The adsorption follows the equation accurately until more than 90 per cent attachment has been accomplished, and then slows down somewhat, indicating either that not all the phage particles have the same affinity for the bacteria, or that equilibrium is being approached. Other experiments not recorded here suggest that, if an equilibrium exists, it lies too far in favor of adsorption to be readily detected. This equation expresses the rate of adsorption even when a tenfold excess of phage over bacteria is present, indicating that a single bacterium can accommodate a large number of phage particles on its surface, as found by several previous workers (5, 10).

Krueger (10) found a true equilibrium between free and adsorbed phage. The absence of a detectable desorption in our case may result from the fixation of adsorbed phage by growth processes, since our conditions permitted growth, whereas Krueger's experiments were conducted at a temperature at which the phage could not grow.

### *Growth of Phage*

Following adsorption of the phage particle on a susceptible bacterium, multiplication occurs, though this is not apparent as an increase in the number of plaques until the bacterium releases the resulting colony of phage particles into the solution. Because the adsorption under proper conditions is so rapid and complete (as shown above) experiments could be devised in which only the influence of the processes following adsorption could be observed.

The details of these experiments were as follows: 0.1 cc. of a phage suspension of appropriate concentration was added to 0.9 cc. of an 18 hour broth bacterial culture, containing about $2 \times 10^8$ *B. coli* / cc. After standing for a few minutes, 70 to 90 per cent of the phage was attached to the bacteria. At this time, the mixture was diluted 50-fold in broth (previously adjusted to the required temperature) and incubated. Samples were removed at regular intervals, and the concentration of infective centers determined.

The results of three experiments at 37°C. are plotted in Fig. 2, and confirm the suggestion of d'Herelle that phage multiplies under a spatial constraint, *i.e.* within or upon the bacterium, and is suddenly liberated in a burst. It is seen that after the initial rise (discussed above) the count of infective centers remains constant up to 30 minutes, and then rises about 70-fold above the initial value. The rise corresponds to the liberation of the phage particles which have multiplied in the initial constant period. This interpretation was verified by measurements of the free phage by centrifuging out the infected bacteria, and determining the number of phage particles in the supernatant liquid. The free phage concentration after adsorption was, of course, small compared to the total and remained constant up to the time of the first rise. It then rose steeply and became substantially equal to the total phage.

The number of bacteria lysed in this first burst is too small a fraction of the total bacteria used in these experiments to be measured as a change in turbidity; the ratio of uninfected bacteria to the total possible number of infected bacteria before the first burst is 400 to 1, the largest number of bacteria which can disappear in the first burst is therefore only 0.25 per cent of the total.

The phage particles liberated in the first burst are free to infect more bacteria. These phage particles then multiply within or on the newly infected bacteria; nevertheless, as before, the concentration of infective centers remains constant until these bacteria are lysed and release the phage which they contain into the medium. This gives the second burst which begins at about 70 minutes from the start of the experiment. Since the uninfected bacteria have been growing during this time, the bacteria lysed in the second burst amount to less than 5 per cent of the total bacteria present at this time. There is again therefore, no visible lysis.

This process is repeated, leading to a third rise of smaller magnitude starting at 120 minutes. At this time, inspection of the culture, which has until now been growing more turbid with the growth of the uninfected bacteria, shows a rapid lysis. The number of phage particles available at the end of the second rise was sufficient to infect the remainder of the bacteria.

These results are typical of a large number of such experiments, at

37°, all of which gave the 70-fold burst size, *i.e.* an average of 70 phage particles per infected bacterium, occurring quite accurately at the time shown, 30 minutes. Indeed, one of the most striking features of these experiments was the constancy of the time interval from adsorption to the start of the first burst. The magnitude of the rise (70-fold) was likewise readily reproducible by all phage preparations which had been stored under proper conditions to prevent deterioration (see above).

### *Multiple Infection*

The adsorption measurements showed that a single bacterium can adsorb many phage particles. The subsequent growth of phage in these "multiple infected" bacteria might conceivably lead to (*a*) an increase in burst size; (*b*) a burst at an earlier time, or (*c*) the same burst size at the same time, as if only one of the adsorbed particles had been effective, and the others inactivated. In the presence of very great excesses of phage, Krueger and Northrop (3) and Northrop (2) report that visible lysis of the bacteria occurs in a very short time. It was possible therefore, that in our case, the latent period could be shortened by multiple infection. To determine this point, we have made several experiments of which the following is an example. 0.8 cc. of a freshly prepared phage suspension containing $4 \times 10^9$ particles per cc. (assay corrected for efficiency of plating) was added to 0.2 cc. of bacterial suspension containing $4 \times 10^9$ bacteria per cc. The ratio of phage to bacteria in this mixture was 4 to 1. 5 minutes were allowed for adsorption, and then the mixture was diluted 1 to 12,500 in broth, incubated at 25°, and the growth of the phage followed by plating at 20 minute intervals, with a control growth curve in which the phage to bacteria ratio was 1 to 10. No significant difference was found either in the latent period or in the size of the burst. The bacteria which had adsorbed several phage particles behaved as if only one of these particles was effective.

### *Effect of Temperature on Latent Period and Burst Size*

A change in temperature might change either the latent period, *i.e.* the time of the burst, or change the size of the burst, or both. In order to obtain more accurate estimates of the burst size it is desirable

to minimize reinfection during the period of observation. This is obtained by diluting the phage-bacteria mixture (after initial contact to secure adsorption) to such an extent that the rate of adsorption then becomes extremely small. In this way, a single "cycle" of growth, (infection, growth, burst) was obtained as the following example

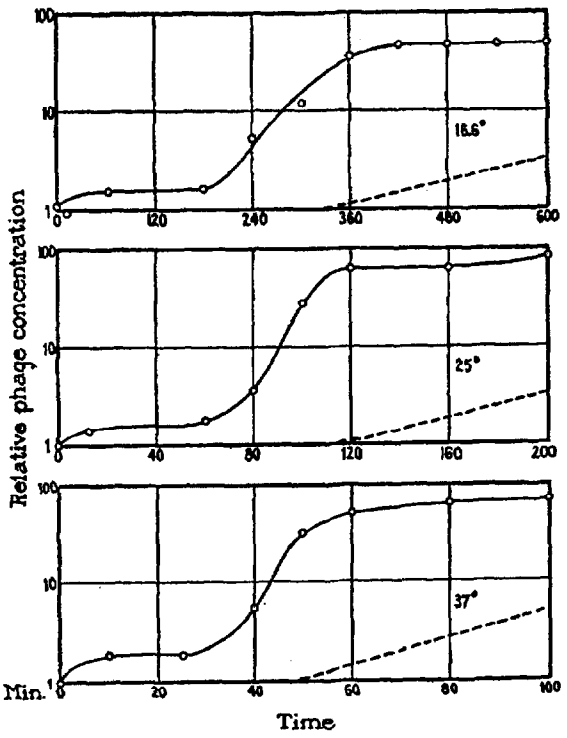


FIG. 3. One-step growth curves.

A suitable dilution of phage was mixed with a suspension of bacteria containing $2 \times 10^8$ organisms per cc. and allowed to stand at the indicated temperature for 10 minutes to obtain more than 90 per cent adsorption of the phage. This mixture was then diluted $1:10^4$ in broth, and incubated. It was again diluted 1:10 at the start of the first rise to further decrease the rate of adsorption of the phage set free in the first step. The time scales are in the ratio 1:2:6 for the temperatures 37, 25, and 16.6°C. Log $P/P_0$ is plotted, $P_0$ being the initial concentration of infective centers and $P$ the concentration at time $t$. The broken line indicates the growth curve of the bacteria under the corresponding conditions.

shows. 0.1 cc. of phage of appropriate and known concentration was added to 0.9 cc. of an 18 hour culture and allowed to stand in this concentrated bacterial suspension for 10 minutes at the temperature of the experiment. This mixture was then diluted $1:10^4$ in broth and incubated at the temperature chosen. Samples of this diluted mixture were withdrawn at regular intervals and assayed. The results of three such experiments are plotted in Fig. 3. The rise corresponds to the average number of phage produced per burst, and its value can be appraised better in these experiments than in the complete growth curve previously given (Fig. 2) where there is probably some overlapping of the steps. In these experiments the rise is seen to be practically identical at the three temperatures, and equals about sixty particles per infected bacterium, but the time at which the rise occurred was 30 minutes at 37°, 60 minutes at 25°, and 180 minutes at 16.6°. This shows that the effect of temperature is solely on the latent period.

We have also made separate measurements of the rate of bacterial growth under the conditions of these experiments. They show that the average division period of the bacteria in their logarithmic growth phase varies in the same way with temperature, as the length of the latent period of phage growth. The figures are:

| Temperature | Division period of *B* | Latent period of *P* growth |
|---|---|---|
| °C | *min.* | *min.* |
| 16.6 | About 120 | 180 |
| 25 | 42 | 60 |
| 37 | 21 | 30 |

There is a constant ratio (3/2) between the latent period of phage growth and the division period of the bacteria. This coincidence suggests a connection between the time required for division of a bacterium under optimum growth conditions, and the time from its infection by phage to its lysis.

### *Individual Phage Particle*

The growth curves described above give averages only of large numbers of bursts. They can, however, also be studied individually, as was first done by Burnett (11).

If from a mixture containing many particles very small samples are withdrawn, containing each on the average only about one or less particles, then the fraction $p_r$ of samples containing $r$ particles is given by Poissons' (12) formula,

$$p_r = \frac{n^r e^{-n}}{r!} \tag{1}$$

where $n$ is the average number of particles in a sample and $e$ is the Napierian logarithm base. If the average number $n$ is unknown, it can be evaluated from an experimental determination of any single

TABLE II

*Distribution of Individual Particles among Small Samples*

A suitably diluted phage preparation was added to 5 cc. of 18 hour bacteria culture and 0.1 cc. samples of this mixture were plated. The distribution of particles among the samples is that predicted by formula (1).

| | $p_r$ (experimental) | $p_r$ (calculated) |
|---|---|---|
| 0 plaques on 13 plates | 0.394 | 0.441 |
| 1 plaque " 14 " | 0.424 | 0.363 |
| 2 plaques " 5 " | 0.151 | 0.148 |
| 3 " " 1 plate | 0.033 | 0.040 |
| 4 " " 0 plates | 0.000 | 0.008 |
| 27 " " 33 " | 1.002 | 1.000 |

one of the $p_r$, for instance from a determination of $p_0$, the fraction of samples containing no particles:

$$n = -\ln p_0 \tag{2}$$

Let us now consider the following experiment. A small number of phage particles is added to a suspension containing bacteria in high concentration. Within a few minutes each phage particle has attached itself to a bacterium. The mixture is then diluted with a large volume of broth, in order to have the bacteria in low concentration so that after the first burst a long time elapses before reinfection, as in the one step growth curves. Samples (0.05 cc.) are removed from this mixture to separate small vials and incubated at the desired temperature. If these samples are plated separately (after adding a drop of bacterial suspension to each vial) before the occurrence of bursts, the fraction of the plates containing 0, 1, 2, *etc.* plaques is found

to conform to formula (1) (see Table II). In this experiment we could also have inferred the average number of particles per sample, using formula (2), from the fraction of the plates showing no plaques (giving 0.93 per sample) instead of from the total number of plaques (27/33 = 0.82 per sample).

### *Experimental Measure of Efficiency of Plating*

If the samples are incubated until the bursts have occurred, and then plated, the samples which had no particles will still show no plaques, those with one or more particles will show a large number, depending on the size of the burst, and on the efficiency of plating. In any case, if we wait until all bursts have occurred, only those samples which really contained no particle will show no plaques, quite independent of any inefficiency of plating. From this fraction of plates showing no plaques we can therefore evaluate the true number of particles originally present in the solution, and by comparison with the regular assay evaluate the efficiency of plating. In this way we have determined our efficiency of plating to be about 0.4. For instance, one such experiment gave no plaques on 23 out of 40 plates, and many plaques on each of the remaining plates. This gives $p_0 = \frac{23}{40}$ or 0.57 from which $n = 0.56$ particles per sample. A parallel assay of the stock phage used indicated 0.22 particles per sample; the plating efficiency was therefore $\frac{0.22}{0.56} = 0.39$. This plating efficiency remains fairly constant under our standard conditions for assay. The increase in probability of plaque formation which we suppose to take place following the infection of a bacterium by the phage particle, *i.e.* the initial rise, brings the plating efficiency up to 0.65.

### *The Burst Size*

Single particle experiments such as that described above, revealed a great fluctuation in the magnitude of individual bursts, far larger than one would expect from the differences in size of the individual bacteria in a culture; indeed, they vary from a few particles to two hundred or more. Data from one such experiment are given in Table III.

We at first suspected that the fluctuation in burst size was connected with the time of the burst, in that early bursts were small and late bursts big, and the fluctuation was due to the experimental superposition of these. However, measurement of a large number of bursts, plated at a time when only a small fraction of the bursts had occurred, showed the same large fluctuation. We then suspected that the particles of a burst were not liberated simultaneously, but over an interval of time. In this case one might expect a greater homogeneity

TABLE III

*Fluctuation in Individual Burst Size*

97.9 per cent of phage attached to bacteria in presence of excess bacteria (10 minutes), this mixture diluted, and samples incubated 200 minutes, then entire sample plated with added bacteria.

| | Bursts |
|---|---|
| 25 plates show 0 plaques | |
| 1 plate shows 1 plaque | 130 |
| 14 plates show bursts | 58 |
| Average burst size, taking account of probable doubles = 48 | 26 |
| | 123 |
| | 83 |
| | 9 |
| | 31 |
| | 5 |
| | 53 |
| | 48 |
| | 72 |
| | 45 |
| | 190 |
| | 9 |
| Total | 882 plaques |

in burst size, if measurements were made at a late time when they are at their maximum value. This view also was found by experiment to be false.

The cause of the great fluctuation in burst size is therefore still obscure.

## DISCUSSION

The results presented above show that the growth of this strain of phage is not uniform, but in bursts. These bursts though of constant

average size, under our conditions, vary widely in individual size. A burst occurs after a definite latent period following the adsorption of the phage on susceptible bacteria, and visible lysis coincides only with the last step-wise rise in the growth curve when the phage particles outnumber the bacteria present. It seemed reasonable to us to assume that the burst is identical with the lysis of the individual bacterium.

Krueger and Northrop (3), in their careful quantitative studies of an anti-*staphylococcus* phage came to an interpretation of their results which differs in some important respects from the above:

1. Their growth curves were smooth and gave no indication of steps; they concluded therefore that the production of phage is a continuous process.

2. In their case, the free phage during the logarithmic phase of a growth curve was an almost constant small fraction of the total phage. This led them to the view that there is an equilibrium between intracellular and extracellular phage. With an improved technique, Krueger (10) found that the fraction of free phage decreased in proportion to the growth of the bacteria, in conformity with the assumption of an equilibrium between two phases.

3. Krueger and Northrop (3) found that visible lysis occurred when a critical ratio of total phage to bacteria had been attained, and they assumed that there was no lysis in the earlier period of phage growth.

To appreciate the nature of these differences it must be born in mind that their method of assay was essentially different from ours. They used, as a measure of the "activity" of the sample of phage assayed, the time required for it to lyse a test suspension of bacteria under standard conditions. This time interval, according to the picture of the growth process given here, is the composite effect of a number of factors: the average time required for adsorption of free phage, its rate of growth in the infected bacteria, the time and size of burst, and the average time required for repetition of this process until the number of phage particles exceeds the number of bacteria and infects substantially all of them. Then, after a time interval equal to the latent period, lysis occurs.

This lysis assay method tends to measure the total number of phage particles rather than the number of infective centers as the

following considerations show. Let us take a sample of a growth mixture in which is suspended one infected bacterium containing fifty phage particles. If this sample is plated, it can show but a single plaque. However, if the sample is assayed by the lysis method, this single infective center soon sets free its fifty particles (or more, if multiplication is still proceeding) and the time required to attain lysis will approximate that for fifty free particles rather than that for a single particle.

Since the burst does not lead to an increase in the number of phage particles, but only to their dispersion into the solution, the lysis method cannot give any steps in the concentration of the *total* phage in a growth curve. On the other hand one might have expected a step-wise increase in the concentration of *free* phage. However, the adsorption rate of the phage used by Krueger (10) is so slow that the infection of the bacteria is spread over a time longer than the presumed latent period, and therefore the bursts would be similarly spread in time, smoothing out any steps which might otherwise appear. Moreover, their measurements were made at 30 minute intervals, which even in our case would have been insufficient to reveal the steps.

The ratio between intracellular and extracellular phage would be determined, according to this picture of phage growth, by the ratio of the average time of adsorption to the average latent period. The average time of adsorption would decrease as the bacteria increased, shifting the ratio of intracellular to extracellular phage in precisely the manner described by Krueger (10).

As we have indicated in the description of our growth curves, lysis of bacteria should become visible only at a late time. Infection of a large fraction of the bacteria is possible only after the free phage has attained a value comparable to the number of bacteria, and visible lysis should then set in after the lapse of a latent period. At this time the total phage (by activity assay) will be already large compared to the number of bacteria, in agreement with Krueger and Northrop's findings.

It appears therefore that while Krueger and Northrop's picture does not apply to our phage and bacteria, their results do not exclude for their phage the picture which we have adopted. It would be of fundamental importance if two phages behave in such a markedly different way.

SUMMARY

1. An anti-*Escherichia coli* phage has been isolated and its behavior studied.

2. A plaque counting method for this phage is described, and shown to give a number of plaques which is proportional to the phage concentration. The number of plaques is shown to be independent of agar concentration, temperature of plate incubation, and concentration of the suspension of plating bacteria.

3. The efficiency of plating, *i.e.* the probability of plaque formation by a phage particle, depends somewhat on the culture of bacteria used for plating, and averages around 0.4.

4. Methods are described to avoid the inactivation of phage by substances in the fresh lysates.

5. The growth of phage can be divided into three periods: adsorption of the phage on the bacterium, growth upon or within the bacterium (latent period), and the release of the phage (burst).

6. The rate of adsorption of phage was found to be proportional to the concentration of phage and to the concentration of bacteria. The rate constant $k_a$ is $1.2 \times 10^{-9}$ cm.$^3$/min. at 15°C. and $1.9 \times 10^{-9}$ cm.$^3$/min. at 25°.

7. The average latent period varies with the temperature in the same way as the division period of the bacteria.

8. The latent period before a burst of individual infected bacteria varies under constant conditions between a minimal value and about twice this value.

9. The average latent period and the average burst size are neither increased nor decreased by a fourfold infection of the bacteria with phage.

10. The average burst size is independent of the temperature, and is about 60 phage particles per bacterium.

11. The individual bursts vary in size from a few particles to about 200. The same variability is found when the early bursts are measured separately, and when all the bursts are measured at a late time.

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